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We have studied, during the course of this project, the regulation and function of the stress-activated protein kinases (SAPKs) and p38s, two subfamilies of the mitogen-activated protein kinases (MAPKs) that directly regulate gene expression, in response to stress and inflammation, by activating components of the activator protein-1 (AP-1) transcription factor. Activation of these pathways may be important in the control of cell death, inflammation and cell cycle progression. Accordingly, exploitation of these pathways might prove useful as a means of inhibiting cancerous growth. Alternatively, inasmuch as the cell death caused by commonly used chemotherapeutics can be attributed in large part to recruitment of AP-1, inhibition of SAPK or p38 might curtail the nonspecific disfiguring and undesirable tissue necrosis that occurs as a side effect of these treatments. We have dissected the framework by which the inflammatory cytokine TNF, which can kill breast cancer cells activates the SAPKs and p38s. This pathway proceeds through TNF receptor associated factor (TRAF)-2 to germinal center kinase (GCK) and receptor interacting protein (RIP). GCK and RIP directly recruit protein kinases upstream of SAPK and p38. We have also shown that activation of the p38 pathway by the Rho family GTPase Cdc42Hs inhibits cell cycle progression.

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FOREWORD

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1. INTRODUCTION

1.1 Background Pertinent to Previous and Ongoing Work

1.1.1 *General Considerations*

Cells respond to extracellular stimuli through the recruitment of signal transduction pathways that in turn act to mount an appropriate response. Pathologic gain of function mutations in growth promoting signaling pathways have been implicated in the pathogenesis of breast cancer. Conversely, environmental stresses, including inflammatory cytokines and commonly used chemotherapeutic agents, activate signaling pathways that arrest cell growth; and can, in some instances, elicit programmed cell death (apoptosis). Exploitation of stress-regulated signaling pathways might therefore be a useful strategy in combating cancer. Alternatively, modulation of the necrotizing actions of cancer therapeutics could reduce the undesirable and disfiguring side effects of these treatments. Our studies focus on two signal transduction pathways recruited by stressful and inflammatory stimuli, the stress-activated protein kinase (SAPK, also called Jun NH_2-terminal kinase, JNK) pathway and the p38 pathway. We believe that dissecting these pathways and elucidating the fundamental biological processes that they govern will lead to the development of novel anti cancer treatment strategies.

1.1.2 *Mitogen-activated protein kinase (MAPK) pathways in eukaryotic cells: an emerging paradigm, the core signaling module*

Signal transduction mechanisms composed of a core of protein Ser/Thr kinases culminating in activation of members of the mitogen-activated protein kinase (MAPK) family have been widely conserved in eukaryotic evolution. All eukaryotic cells possess multiple MAPK pathways poised to respond preferentially to distinct extracellular inputs. The existence of multiple parallel pathways allows a cell to respond simultaneously to different classes of stimuli [1,2].

At the heart of all MAPK pathways is a central, three tiered core module of protein kinases wherein the MAPKs are activated by concomitant Tyr and Thr phosphorylation catalyzed by members of the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) family. MEKs, in turn, are activated by Ser/Thr phosphorylation catalyzed by several protein kinase families collectively termed MAPK kinase-kinases (MAP3Ks) [1,2]. A diverse variety of molecular species has been implicated in the regulation of MAP3K → MEK → MAPK core pathways. In mammals, these include small GTPases of the Ras superfamily as well as protein kinases of the Ste20 and Sps1 families, and adaptor proteins coupled to cytokine receptors [1,2]. The best characterized mammalian MAPK pathway is the Ras-MAPK pathway wherein mitogen receptors, such as those for EGF or insulin, recruit Ras which, in turn elicits activation of the MAP3K Raf-1. Raf-1 activates the mitogenic MEKs MEK1 and MEK2 which then activate the p42 and p44 MAPKs [3]. Whereas this mitogenic MAPK pathway has been elucidated in good detail; in general, the regulation of most mammalian MAP3Ks and, by extension, most MAPK core signaling modules is poorly understood.

1.1.3 *The SAPK and p38 pathways: mammalian MAPK pathways activated by stress and inflammatory cytokines*

The SAPKs and the p38s are two recently described MAPK subfamilies activated preferentially by environmental stresses. The SAPKs and p38s are poorly activated by mitogens such as EGF or insulin [1,4-9]. Of particular importance, the SAPKs and p38s are major targets of two inflammatory cytokines: TNF and IL1 [1,4-9]. In response to stressful and inflammatory stimuli, the SAPKs and p38s can regulate gene expression through the

direct phosphorylation and activation of transcription factors. The SAPKs and p38s are crucial elements in the program of TNF-induced gene expression, and therefore to the physiology of TNF, in their capacity as the dominant kinases responsible for activation of the activator protein-1 (AP-1) transcription factor [4,5,10].

AP-1 typically consists of a heterodimer of c-Jun and a member of the c-Fos or activating transcription factor (ATF) families [10]. AP-1 dimers bind to a *cis* acting element, the TPA response element (TRE), in the promoters of stress and mitogen-regulated genes. Activation of AP-1 *trans* activating activity and DNA binding, either through phosphorylation of, or elevations in the levels of AP-1 constituents, in turn, recruits the transcriptional machinery [10]. Both the SAPKs and p38s can regulate AP-1 at several levels. SAPK phosphorylation of c-Jun within the *trans* activation domain (Ser 63 and 73) correlates well with elevated c-Jun/AP-1 *trans* activating activity [4,5,10]. SAPK phosphorylation of the transcription factor Elk1 at Ser 383 and 389 activates ternary complex factor function which, through its association with the serum response factor, participates in the induction of c-*fos* [11]. The SAPKs and p38s can also phosphorylate ATF2 at Thr 69 and Thr 71 thereby activating its *trans* activating function [12]. Finally, p38 can phosphorylate (at Thr 293 and 300) and activate the *trans* activating function of myocyte enhancer factor-2C (MEF2C), a transcription factor implicated in stress and cytokine induction of c-*jun* [13]. AP-1 activation by inflammatory cytokines such as TNF and IL1 is crucial to the subsequent induction of additional cytokines, such as IL2 and IL6 as well as additional TNF, and the expression of proteases such as collagenase, that target the extracellular matrix. All of these factors contribute to implementation of the inflammatory response [14-17]. In addition, recent studies have shown that c-Jun and AP-1 activation is critical to apoptosis [18].

In addition to activation of transcription factors, p38 can also activate other protein kinases. Thus, p38 can phosphorylate and activate MAPK-activated protein kinase-2 (MAPKAP-K2), a Ser/Thr kinase that, in turn, can phosphorylate the small heat shock protein Hsp25. Phosphorylation of Hsp25 has been implicated in growth arrest and apoptosis [7,8].

The SAPKs contain a characteristic -T_{183}-P-Y_{185}- motif within the L12 loop of subdomain VIII of the catalytic domain [1,4,5]. Phosphorylation of T_{183} and Y_{185} results in SAPK activation and is catalyzed by at least two MEKs, SAPK/ERK kinase-1 (SEK1) and MAPK-kinase (MKK)-7 [19,20]. The PI collaborated with Dr. Leonard Zon to identify SEK1 as a SAPK activator [19]. Similarly, the p38s contain the characteristic phosphorylation loop T-G-Y. Tyr and Thr phosphorylation at these sites results in p38 activation and is catalyzed by at least two MEKs, MKK3 and MKK6 [1,6-8,21,22].

Several protein kinase families have been implicated as MAP3Ks upstream of the SAPKs and p38s. The MEK-kinases (MEKKs) are mammalian homologues of, a MAP3K of the budding yeast *Saccharomyces cerevisiae*, that regulates both the mating pheromone and osmosensing pathways [1,2,23]. Mammalian MEKKs include MEKKs-1-4, apoptosis signal-regulating kinase-1 (ASK1) and TGF-β-activated kinase-1 (TAK1) [24-32]. In collaboration with Dr. Dennis Templeton's laboratory, we showed that MEKK1 could phosphorylate and activate SEK1 [26]. MEKK1 is entirely SAPK specific; and under physiologic circumstances it cannot activate either the mitogenic MAPK pathway or the p38 pathway [1,26]. MEKK4, ASK1 and TAK1 can each activate both the SAPK and p38 pathways *in vivo* and can activate SEK1, MKK3 and MKK6 *in vitro* and *in vivo* [28-32]. MEKKs-2 and -3 can activate both the SAPK pathway (via SEK1) and the mitogenic MAPK pathway (via MEK1-dependent and -independent mechanisms) [27]. Mixed lineage kinases

(MLKs) have also been implicated as MAP3Ks. These kinases bear structural homology to both Ser/Thr and Tyr kinases. MLK2, MLK3 are SAPK-specific while dual lineage kinase (DLK) can activate both the SAPK and p38 pathways [33-35].

1.1.4. *Coupling SAPK and p38 core signaling modules to upstream components: Rho family GTPases and Mammalian Sps1s*

The diversity of MAP3Ks upstream of the SAPKs and p38s is a reflection of the diversity of agonists that recruit these pathways. Consistent with this diversity, a broad array of upstream elements feeds into SAPK and p38 core signaling modules. These include the Rho family GTPases Rac1 and Cdc42Hs. Rho GTPases are a subset of the Ras superfamily of monomeric GTPases. These proteins act as molecular switches, active in the GTP bound state and inactive in the GDP bound state [1,3,36]. GTP-loaded Ras family proteins bind effector proteins and so propagate signals from the membrane to the inside of the cell. Upstream activators of Ras type GTPases promote the exchange of GDP for GTP, a reaction catalyzed by guanine nucleotide exchange factors (GEFs). GEFs for Ras include mammalian son of sevenless (mSOS). GEFs for Rho family GTPases include members of the Dbl proto oncogene family [3,36]. Inactivation of Ras proteins is mediated by a slow intrinsic GTPase activity greatly accelerated by GTPase activating proteins (GAPs) [3,36].

Constitutively active (GTPase-deficient) Rac1 and Cdc42Hs can activate both the SAPKs and p38s [36-38]. However, the effectors that couple these G proteins to the SAPKs and p38s remain to be unambiguously identified, inasmuch as several upstream activators of the SAPKs and p38s, including MEKK4, MLK3 and kinases of the p21-activated kinase (PAK) family, can bind GTP-charged Rac1 and Cdc42Hs [28,39-41]. RhoA, Rac1 and Cdc42Hs have also been implicated in the regulation of the actin cytoskeleton. Specifically, RhoA promotes the formation of actin stress fibers, Rac1 promotes lamellopodium formation and Cdc42Hs promotes the formation of actin filopodia. The significance of these phenomena is unclear; however, genetic and molecular biological studies have shown that Rho, Rac1 and Cdc42Hs are components in serum stimulated cell cycle entry and are possibly effectors for Ras transformation.

Mammalian homologues of the *S. cerevisiae* protein kinase *SPS1* [42] act independently of Rho family GTPases to recruit the SAPKs. Mammalian Sps1s consist of an amino terminal kinase domain, distantly related to those of the PAKs, and an extended carboxyterminal regulatory domain. The regulatory domains of the different Sps1s are highly divergent [1,42]. The first of the mammalian Sps1s to be identified was germinal center kinase (GCK). GCK is ubiquitously expressed; however, in B follicular tissue, its distribution is restricted to the germinal center. B cell selection and maturation occur in the germinal center and are mediated in part by receptors of the TNFR family including TNFR1, CD40, CD30 and CD27 [43].

Given the segregation of GCK in the germinal center, and the involvement of TNFR1 and CD40, both of which recruit the SAPKs [4,44], in B cell selection, we proposed that GCK might itself be regulated by TNF-like receptors and might couple to the SAPKs. We have since shown that endogenous GCK is activated *in vivo* by TNF [45]. Moreover, expression of GCK results in a dramatic activation of the SAPK pathway [45]. The p38s and the MAPKs are not activated [45]. Taken together, these results suggest that GCK is an important effector for coupling TNF receptors to the SAPKs. GCK is constitutively active upon overexpression, an indication that it is regulated by aggregation or by limiting concentrations of an inhibitor, both of which are mechanisms that could be overcome by overexpression. Consistent with a role for the GCK-C-terminus in GCK regulation, we

observe that expression of the GCK-C-terminal noncatalytic domain can activate the SAPK pathway, albeit to a much smaller extent than can wild type GCK [45].

Subsequently, two other mammalian Sps1s, hematopoietic progenitor kinase-1 (HPK1) and Nck interacting kinase were shown to activate selectively the SAPK pathway [46-48]. HPK1 can interact with both MEKK1 and MLK3, and kinase inactive mutants of these MAP3Ks can inhibit HPK1 activation of the SAPKs. Thus it is likely that MLK3 and MEKK1 are HPK1 targets *in vivo* [46,47]. Nck interacting kinase can also interact with MEKK1; and kinase-dead MEKK1 can block Nck interacting kinase activation of the SAPKs. Thus, it is likely that MEKK1 is also a target of Nck interacting kinase [48]. We too see an interaction between MEKK1 and GCK (see section 2.5).

1.1.5 *Signaling to the SAPKs and p38s through the type-1 TNF receptor*

TNF can promote the apoptotic death of numerous cell types, including breast cancer cells [14]. The SAPKs are potently activated by TNF and several recent studies have begun to identify signaling components that couple the TNF receptors (TNFRs) to the SAPKs. TNF is a homotrimeric ligand, and TNF binding results in receptor trimerization [49]. A significant advancement in the understanding of TNF signaling came with the discovery of polypeptide species that are recruited to the TNFRs as a result ligand-induced receptor trimerization. Many of these proteins, upon overexpression, can, in a ligand-independent manner, elicit the cellular responses to TNF. The identification of these signal transducers has given rise to the protein recruitment model for TNFR signaling wherein occupancy of the TNFR promotes the binding of signal transducing polypeptides which then relay signals to downstream effectors.

The TNFRs are part of a large family of receptors that share homology within their extracellular domains, but are divergent within their intracellular extensions [49]. Many of these receptors (notably Fas and the type-1 TNF receptor [TNFR1]) contain an ~80 amino acid death domain [50-52]. The death domain is necessary for these receptors to elicit numerous biological responses, including apoptosis. Death domains mediate homotypic and heterotypic protein-protein interactions, thereby coupling receptors to their effectors [50-52]. Thus the death domain-containing polypeptide TNFR associated death domain protein (TRADD) can bind TNFR1 [52].

TRADD can also bind a second polypeptide, TNF receptor-associated factor-2 (TRAF2). The TRADD-TRAF2 interaction serves to recruit TRAF2 to TNFR1 [53-55]. The TRAFs are an emerging class of signal transducers important in TNF family signaling. TRAF polypeptides generally contain carboxyterminal TRAF domains and amino terminal "really interesting new gene" (RING) and Zn-finger domains. TRAF domains, like death domains, mediate homotypic and heterotypic protein-protein interactions [53-55]. The association between TRADD and TRAF2 requires the C-terminal (amino acids 356-501) of two TRAF domains on TRAF2 and a TRAF binding domain on TRADD (amino acids 106-169) [54,55]. The TRADD-TRAF2 interaction is necessary for coupling TNFR1 to activation of the nuclear factor-κB (NF-κB) transcription factor [52-55]. NF-κB is required for much of the gene expression elicited by TNF [14,51].

Overexpression of TRAF2 not only results in activation of NF-κB, but can also promote potent SAPK and AP-1 activation [56,57], as well as activation of the p38 pathway (see section 2.7). Mutant TRAF2 constructs devoid of the RING finger domain sequester TRADD preventing binding of endogenous TRAF2. By this process, these mutants can inhibit TNF activation of the SAPKs [53,55-57].

Receptor interacting protein (RIP) is a second species which, like TRAF2, can be recruited, in a TNF-dependent manner, to TNFR1 through an interaction with TRADD

[58,59]. RIP contains an amino terminal protein kinase domain (amino acids 1-305), an intermediate domain (amino acids 306-553) and a death domain (amino acids 554-656). The latter mediates the TRADD-RIP interaction [58,59]. RIP can also interact with TRAF2 via the RIP intermediate domain and kinase domains and the TRAF2 C-terminal TRAF domain [55,58,59]. RIP overexpression can activate NF-κB and can also activate the SAPK pathway [56,60]. In addition, we have observed activation of the p38 pathway by overexpressed RIP (see section 2.2). Expression of the RIP death domain can block SAPK activation by TNF likely through the sequestration of endogenous TRADD--preventing access to TRADD by either TRAF2 or RIP [56]. The mechanisms by which RIP and TRAF2 couple to the SAPK and p38 pathways are unclear.

1.2 Subject, Purpose of Research

Our interest is to identify and elucidate the biochemistry and cell biology of the SAPK and p38 pathways, signal transduction mechanisms activated by stress and inflammatory cytokines. Our ongoing experiments indicate that these pathways may inhibit cell growth and could thereby counteract the transformation process. In addition, these pathways may actually promote apoptosis [14,18]. Once these pathways are understood, therefore, manipulation of stress signaling at the bedside, through novel therapeutic techniques, could prove efficacious in (1) limiting tumor growth through activation of stress signaling, or, conversely, (2) limiting the toxicity of genotoxic cancer chemotherapeutics by selectively inhibiting their activation of SAPK and p38.

1.3 Scope of Research (8/94-8/98)

The following list is a brief summary of the significant research accomplishments from this laboratory that were supported by the U.S. Army Breast Cancer Grant.

1) We have identified SEK1 as an immediate upstream activator of the SAPKs.

2) We have shown that SEK1 can be phosphorylated and activated by MEKK1.

3) We have shown that the SAPK pathway can be activated by GCK *in vivo*.

4) We have demonstrated that GCK can bind both MEKK1 and TRAF2, thereby linking the TNFR1 signaling complexes to the SAPKs.

5) We have shown that RIP activates the SAPK and p38 pathways *in vivo*, possibly as an effector for TRAF2, and can associate with an endogenous MAP3K upstream of the SAPKs.

6) We have shown that cells in the G1 phase of the cell cycle can be induced to arrest growth at G1/S upon expression of elements of the p38 pathway.

7) We have demonstrated that this cell cycle arrest can be mediated by the Rho family GTPase Cdc42Hs.

2. BODY

This section summarizes and describes key data pertinent to the research accomplishments described in section 1.3

2.1 MAPKAP kinase-2 (MAPKAP-K2) is Not a SAPK Substrate.

During the early stages of this project, we were interested in determining if MAPKAP-K2 was a SAPK substrate. MAPKAP-K2 has been shown to be the principal kinase responsible for phosphorylation of Hsp25, a reaction that correlates with cell growth arrest and apoptosis [1,7,8]. We used purified proteins (purified MAPKAP-K2 was provided by Prof. Philip Cohen) to demonstrated that SAPK cannot phosphorylate and

activate MAPKAP-K2. Fig. 1 illustrates these results. Subsequently, it was shown by Prof. Cohen and others that p38 was the physiologically relevant stress-activated MAPKAP-K2 kinase [7,8].

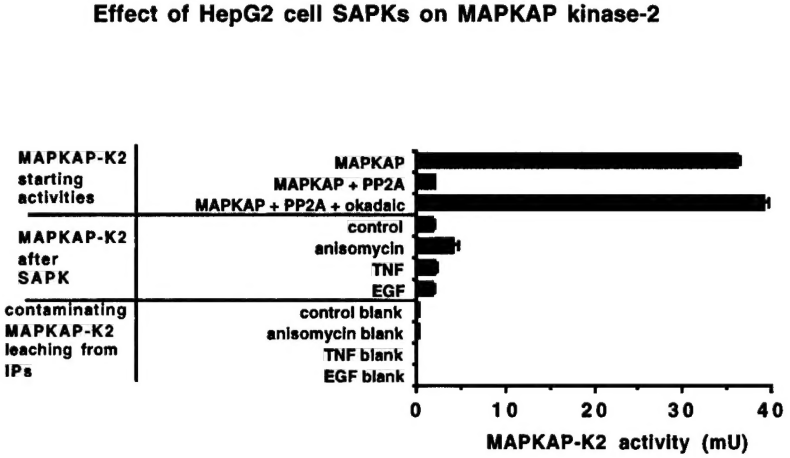

Fig. 1. SAPK does not reactivate phosphatase-2A-inactivated MAPKAP-K2. SAPK was immunoprecipitated from HepG2 cells treated with the ligands indicated and incubated with purified MAPKAP-K2 that had been inactivated with phosphatase-2A as indicated. After centrifugation to remove the SAPK beads, MAPKAP-K2 was assayed as described.

2.2 Identification of SAPK/ERK Kinase-1 (SEK1) as an Upstream Activator of the SAPKs.

Using recombinant SAPK as a substrate, the PI-s laboratory has identified a novel member of the mitogen-activated protein kinase/extracellular signal regulated kinase-kinase (MEK) family, SEK1, as an upstream activator of the SAPKs. SEK1 was cloned in the laboratory of Dr. Leonard Zon, with whom we collaborated in these studies. SEK1 is completely specific for the SAPKs, being unable to activate the MAPKs *in vivo* or *in vitro*. SEK1 activity is activated preferentially by the same stress stimuli that activate the SAPKs *in vivo*. These results further illustrate the segregation between the mitogen-activated Ras/MAPK pathway and the SAPK stress-regulated pathway [1,19]; and support the contention that mammals, like yeast possess multiple, homologous signaling pathways that respond to distinct types of extracellular stimuli. Fig. 2 illustrates activation of the SAPKs *in vitro* by purified SEK1 [19].

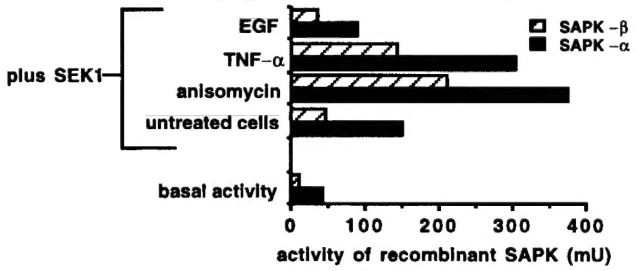

Fig. 2. Activation of SAPKs by SEK1. SEK1 was purified from transfected cells and assayed for activation of purified recombinant SAPKs p54α1 and p54β1. Assays were performed as in Sánchez, *et al.* [19].

2.3 Identification of MEK-kinase-1 (MEKK1) as an Upstream Activator of SEK1 and the SAPK Pathway.

MEKK1 is a mammalian homologue of the yeast MEK activators Ste11 and Byr2 [2,24,25]. Originally, MEKK1 was thought to act as a mitogen-activated, Raf-1-independent mechanism of MEK activation [24]. Using an inducible MEKK1 construct, in collaboration with Dr. Dennis Templeton, we have demonstrated that MEKK1 preferentially activates the SAPK pathway, via activation of SEK1 [26]. These studies, further delineate the segregation of the SAPK and MAPK pathways and strikingly illustrate the homology between yeast and mammalian signaling mechanisms. Fig. 3 illustrates *in vivo* activation of SAPK upon coexpression with MEKK1.

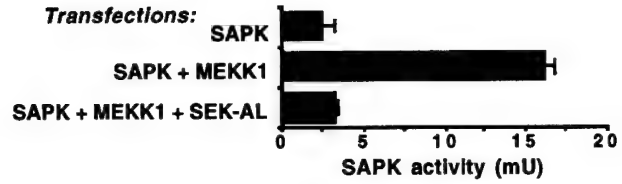

Fig. 3. Coexpression with MEKK1 activates SAPK. This activation is blocked by triple transfection with a nonactivatable SEK1 mutant.

Fig. 4 illustrates the ability of purified MEKK1, *in vitro* to activate the SAPK activating activity of SEK1.

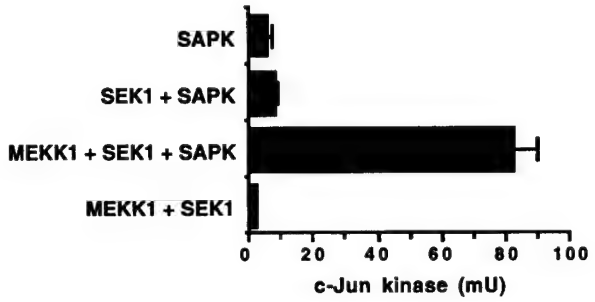

Fig. 4. MEKK1, SEK1 and SAPK-p54α1 were expressed as GST fusion proteins in *E. coli* and purified by glutathione affinity chromatography. Purified MEKK1 was then tested for its ability to activate SEK1 SAPK activating activity in this coupled assay.

2.4 Identification of Germinal Center Kinase (GCK), a Mammalian *SPS1* Homologue, as an Activator of the SAPK Pathway.

In yeast, the MEKK → MEK → ERK core kinase modules are thought themselves regulated by members of the *STE20* and *SPS1* families of protein kinases [2]. Germinal center kinase (GCK) is a ubiquitously expressed mammalian *SPS1* homologue which, in lymphoid follicles is thought to participate in B cell maturation [43]. We have shown that GCK is a potent, specific *in vivo* activator of the SAPK pathway. Expression of GCK activates both SEK1 and the SAPKs. Figs. 5 and 6 illustrate activation of SAPK and SEK1, respectively upon coexpression with GCK. In Fig. 7, it is clear that GCK does not activate two other ERK pathways, the p42/p44 MAPK or p38/mpk2 pathway [45].

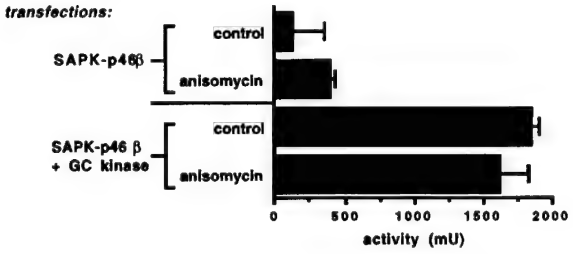

Fig. 5. Activation *in vivo* of SAPK by GCK. 293 cells were transfected with HA-tagged SAPK and GCK or the cognate empty plasmid. SAPK was immunoprecipitated and assayed.

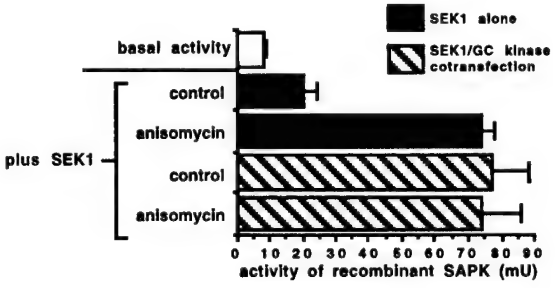

Fig. 6. Activation *in vivo* of SEK1 upon coexpression with GCK. 293 cells were transfected with GST-tagged SEK1 and either GCK or cognate empty plasmid. SEK1 was purified by glutathione agarose affinity chromatography and assayed for activation of SAPK.

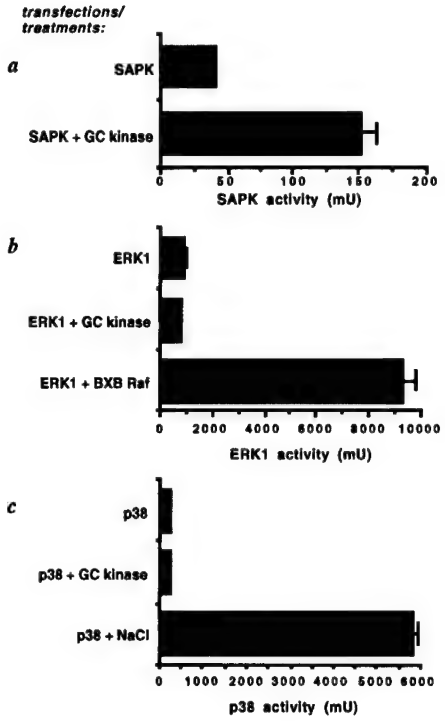

Fig. 7. Coexpression with GCK in COS cells activates the SAPK pathway (part *a*) but does not activate the p42/p44 MAPK pathway (part *b*, ERK1) or the p38 pathway (part *c*) in spite of the fact that these two pathways are intact and can be activated, respectively by oncogenic Raf-1 (BXB-Raf) or by hyperosmotic shock (NaCl).

2.5 GCK is a Regulated Scaffold Protein That Binds MEKK1

Both GCK and MEKK1 show a strong preference for activation of the SAPK pathway via activation of SEK1 [1,26,45]. p38 and MAPK are not activated by either except under conditions of massive overexpression [1,26,45]. This observation led us to ask if MEKK1 was a GCK target and if GCK could physically associate with MEKK1. GCK consists of an amino terminal kinase domain and an extensive carboxy terminal regulatory domain, (CTD, Fig. 10, left panel). We expressed in 293 cells gluathione-*S*-transferase (GST)-tagged constructs of full length GCK or the GCK-CTD. The GST polypeptides were isolated on glutathione (GSH) agarose and probed with an antibody to MEKK1 to detect any endogenous MEKK1 bound to the GCK. Fig. 8 shows that endogenous MEKK1 can associate *in vivo* with GCK, preferentially interacting with the GCK CTD. Binding to full length GST-GCK or GST alone is not detected in this assay.

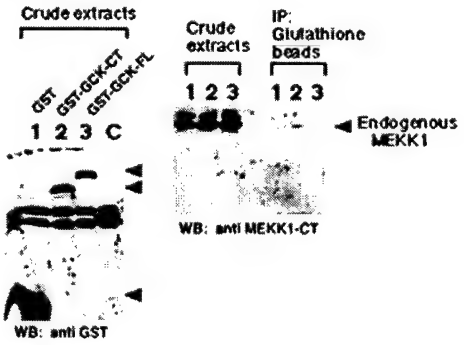

Fig. 8. Interaction between the GCK C-terminal domain and endogenous MEKK1. The numbers indicate the GST constructs transfected into the 293 cells. Left panel: crude extracts blotted with anti GST. Arrowheads indicate, top to bottom, GST-GCK, GST-GCK-CT, GST. Right panel: left three lanes, crude extracts blotted with anti-MEKK1, right three lanes GST isolates blotted with anti MEKK1 antibody. The MEKK1 band is indicated with an arrow. Cells were transfected with the indicated GST-tagged constructs. GSH isolates were subjected to SDS-PAGE and immunoblotting with anti MEKK1 antibody.

We sought next to determine if recombinant MEKK1 displayed a similar preference for the GCK-CTD. In addition, we wished to determine the region of the MEKK1 polypeptide necessary for GCK binding. The MEKK1 polypeptide is quite large (130-kDa)

and consists of a carboxyterminal kinase domain (AAs 1221-1493) and an extensive amino terminal domain (AAs 1-1221) [24,25]. The MEKK1 amino terminal region contains several motifs suggestive of complex regulation. These include two Src homology 3 (SH3) binding domains (AAs 74-149 and 233-291) and two plekstrin homology (PH) domains (AAs 439-555 and 643-750), which may mediate membrane association [25]. In addition, MEKK1 contains an acid-rich segment (AAs 817-1221) in its amino terminus [24,25]. Inasmuch as MEKK1 constructs wherein the amino terminus has been totally or partially deleted are constitutively active [24,26], it is likely that the MEKK1 amino terminal domain exerts a negative regulatory effect on MEKK1. We expressed GST-GCK or GST-GCK-CTD with either of three M2-FLAG-tagged MEKK1 constructs: MEKK1 with a partial amino terminal deletion (AAs 817-1493--this deletes both PH domains and the SH3 binding sites [25]), this same construct with part of the catalytic domain (subdomains V-XI) deleted (AAs 817-1340), or all of the catalytic domain deleted (AAs 817-1221) [25]. Reciprocal anti FLAG immunoprecipitation/anti GST immunoblots (to detect GCK bound to immobilized MEKK1) or GST pulldowns/anti FLAG immunoblots (to detect MEKK1 bound to immobilized GCK) were performed. Fig. 9 shows that all three MEKK1 constructs bind GCK equally well and, like endogenous MEKK1, interact more strongly with the GCK-C-terminal regulatory domain. We do not observe a strong interaction between GCK and other MAP3Ks implicated in SAPK regulation, including ASK1, MLK2 and MLK3.

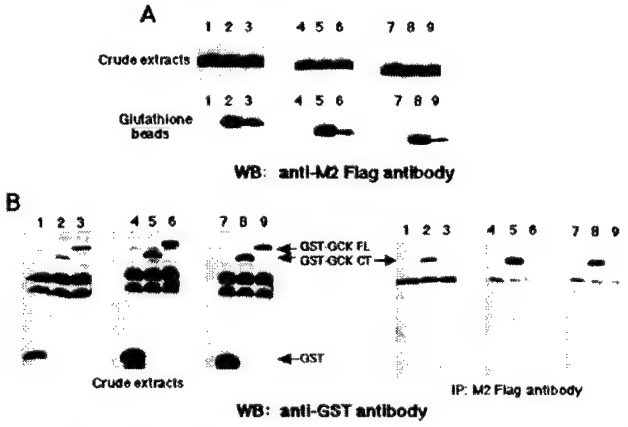

Fig. 9. Recombinant MEKK1 preferentially binds the GCK-CT. The domain on MEKK1 that binds GCK is an acid-/Pro-/Ser/Thr-rich region of the MEKK1 amino terminal regulatory domain (AAs 817-1221). The MEKK1 catalytic domain is not required for binding GCK. 293 cells were transfected with the indicated GST-GCK and M2-FLAG MEKK constructs. (A) Extracts and GSH agarose isolates were probed with anti-M2-FLAG to detect bound MEKK1. (B) Extracts and M2-FLAG immunoprecipitates were probed with anti GST antibody to detect GCK. Lanes 1-3 transfection with MEKK1 817-1493; lanes 4-6 MEKK1 817-1340); lanes 7-9 MEKK1 817-1221. Lanes 1,4 and 7, GST only; lanes 2, 5 and 8, GST-GCK-CT; lanes 3,6 and 9, GST-full length GCK.

We next wished to map more precisely the region of the GCK-CT that binds MEKK1. The GCK-CTD consists of three PEST motifs (PEST 1, 2 and 3; Fig. 10, left panel), a leucine-rich domain and a short C-terminal extension, the CT [43]. M2-FLAG-tagged constructs, wherein these subdomains of the GCK-CTD were progressively deleted, were expressed in 293 cells with GST-MEKK1. In addition, we assessed the binding of MEKK1 to a kinase-dead (K44M) GCK construct in which the Lys residue in the ATP binding domain was mutated to Met. Deletion of the CT abrogates MEKK1 binding completely. Subsequent deletion of the Leu-rich domain restores binding while deletion of PEST3 again results in a GCK construct incapable of binding MEKK1. We tentatively conclude that PEST3 and the CT are necessary for binding. CT may serve to relieve an inhibition to binding conferred by the Leu rich domain.

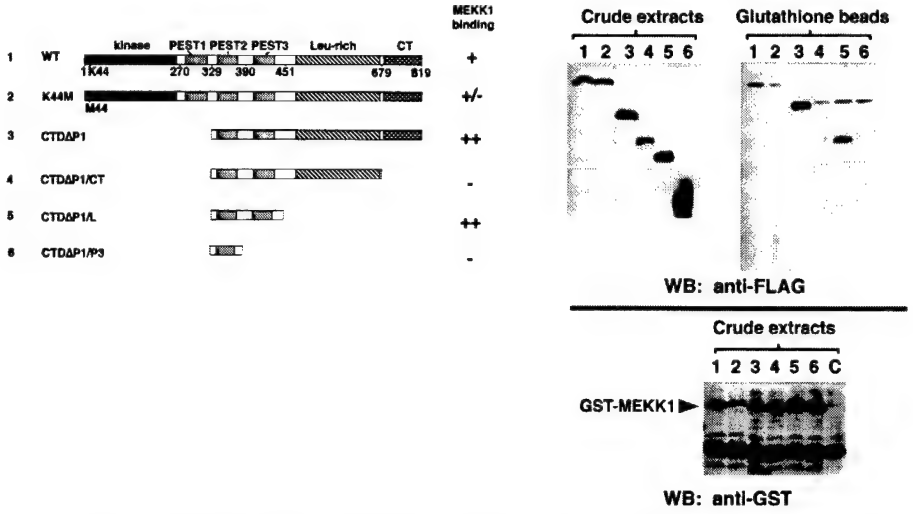

Fig. 10. Binding of GCK to MEKK1 requires PEST3 of the GCK-CT. (left) Schematic illustration of the GCK constructs used. The numbers 1-6 refer to the lanes in gels shown on the right. 293 cells were cotransfected with the indicated M2-FLAG-tagged GCK constructs and GST-tagged MEKK1. Extracts or GSH agarose isolates were immunoblotted with anti FLAG to detect expression of or MEKK1 binding, respectively, of the various GCK constructs.

From Fig. 10, it is also clear that kinase-dead (K44M) GCK, while still able to bind MEKK1 does so even more weakly than does wild type. As before, the GCK-CTD binds MEKK1 most strongly. By contrast, we consistently observe that wild type GCK is the most potent SAPK activator, although both K44M-GCK and the GCK-CT can engender substantial SAPK activation [45]. The simplest reconciliation of these results is that activation of GCK's kinase activity promotes MEKK1 binding and mediates a more rapid turnover of MEKK1, thereby allowing for efficient SAPK pathway activation.

In order to demonstrate the functional significance of the GCK-MEKK1 interaction, we performed two experiments. First, we assessed the ability of GCK constructs devoid of PEST3 to activate the SAPKs. Second, we tested the ability of the GCK binding domain of MEKK1 to inhibit GCK activation of coexpressed SAPK. From Fig. 11, it is clear that deletion of PEST3 (ΔP3, Fig. 11), which abrogates completely MEKK1 binding (Fig. 10), also significantly compromises GCK activation of coexpressed SAPK.

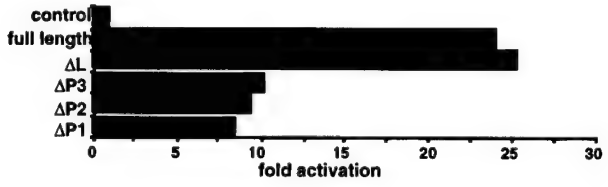

Fig. 11. *In vivo* activation of the SAPK pathway by M2-FLAG-GCK deletion constructs. GCK constructs were expressed in 293 cells with HA-SAPK (p46-β1 isoform). SAPKs were subjected to IP and assay for c-Jun kinase activity. P refers to PEST domains, L, the Leu-rich domain.

In addition, expression of MEKK1ΔC (residues 817-1340) can strikingly inhibit GCK activation of coexpressed SAPK (Fig. 12). Inasmuch as this MEKK1 construct binds GCK (Fig. 9), but is devoid of substrate binding domains, does not inhibit GCK's kinase activity (Fig. 12) and cannot bind SEK1 or activate coexpressed SAPK; it is likely that MEKK1ΔC acts by sequestering the expressed GCK and preventing binding of endogenous MEKK1.

The interaction between GCK and MEKK1 is quite stable--even that between full length GCK and MEKK1, while comparatively weaker than the GCK-CTD-MEKK1 interaction, is still resistant to stringent washing (1M LiCl and 1% Triton X-100, Fig. 9). Moreover, once GCK has bound MEKK1, GCK can phosphorylate the GCK binding domain on the MEKK1 polypeptide (Fig. 13). In the experiment shown in Fig. 13, soluble GST-GCK

was added to immunoprecipitates of a MEKK1 construct (AAs 817-1221) devoid of a catalytic domain (and therefore phosphotransferase activity) or to blank beads. After a 20 min incubation, the beads were washed to remove unbound GCK and the remaining GCK-MEKK1 complexes incubated with γ-^{32}P-ATP. What is noteworthy in Fig. 13 is that a significant portion of the GCK added in the experiment is retained on the MEKK1 beads, even after extensive washing. Thus, GCK-MEKK1 complexes can be generated *in vitro*. From the data in Figs. 1-6 we conclude that MEKK1 is a physiologic target of GCK.

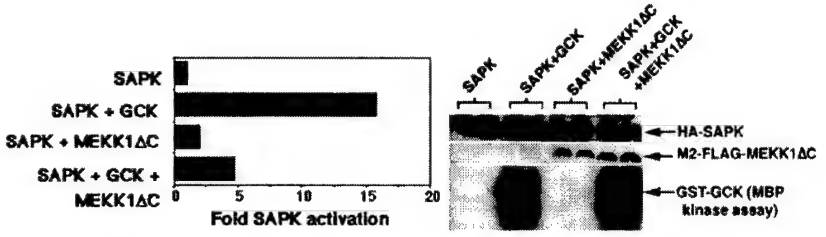

Fig. 12. MEKK1ΔC blocks GCK activation of the SAPKs. 293 cells were transfected with GST-GCK, HA-SAPK or M2-FLAG-MEKK1ΔC as indicated. SAPK was immunoprecipitated and assayed for c-Jun kinase (left panel). Extracts were probed for expression of the indicated constructs (right panel). GCK was assayed for MBP kinase due to the low amount of GCK transfected in this experiment.

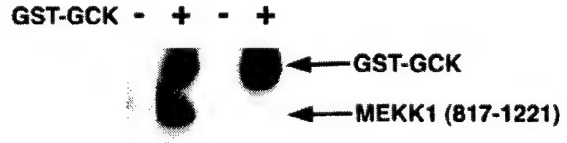

Fig. 13. GCK can phosphorylate the PEST-rich GCK binding domain of MEKK1. 293 cells were transfected with M2-FLAG-MEKK1 (817-1221). GST-GCK was purified from transfected 293 cells by GSH chromatography and elution with free glutathione. MEKK1 (817-1221) was immunoprecipitated and incubated with vehicle or purified GCK and ^{32}P-ATP as indicated. As a control, blank beads (no antibody) were used (far right lane only, all other lanes contained MEKK1 [817-1221]). In the far right lane, the beads were subjected to SDS-PAGE without washing in order to monitor GCK autophosphorylation as a verification of activity. The MEKK1 beads were washed (3 X 1 M LiCl) and subjected to SDS-PAGE. The autophosphorylated GCK and phosphorylated MEKK1 polypeptides are indicated with arrows.

2.6 GCK binds TRAF2 and May Couple the TNFR1 Signaling Complex to the SAPKs

We next sought to determine if GCK was capable of recruiting MEKK1 to the TNFR signaling complex. We have observed that GCK is activated *in vivo* by TNF [45]. TNF signaling to the SAPK pathway employs both TRAF2 and RIP [56,57]. We observe a physical association between TRAF2 and the GCK-CTD (Fig. 14). We do not observe an association between RIP and GCK.

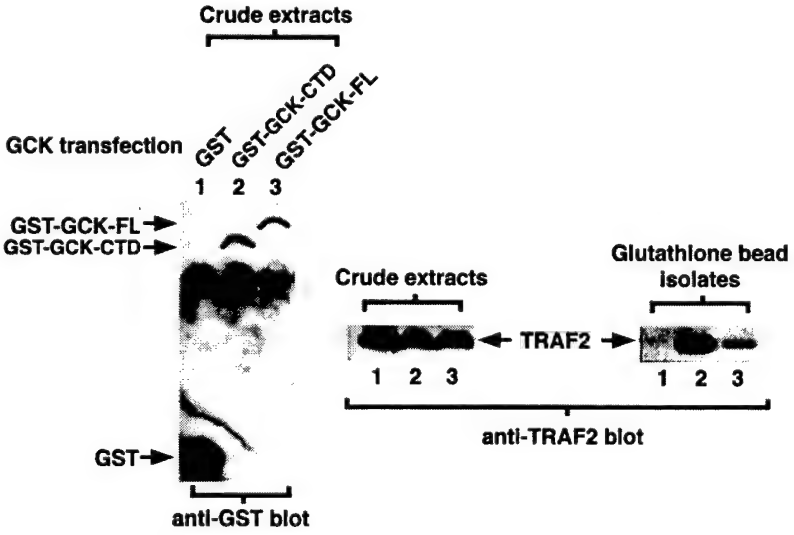

Fig. 14. Association between GCK and TRAF2. 293 cells were transfected with TRAF2 (untagged) and the indicated GST-tagged GCK constructs. GST-tagged polypeptides were purified on GSH agarose and probed in immunoblots with anti TRAF2 antibody. Left seven lanes are crude extracts probed with anti GST antibody or anti TRAF2 antibody as indicated. The right four lanes are an anti TRAF2 blot of GST pulldowns. The numbers refer to the GCK construct transfected.

The results in Fig. 14 indicate that GCK can couple to the TNFR signaling complex by associating with TRAF2. Again, the GCK C-terminal tail binds TRAF2 more stably than does full length GCK. As was the case with MEKK1 binding, activation of GCK's kinase activity, possibly as a result of binding TRAF2, might promote the rapid turnover of TRAF2-GCK complexes *in vivo,* thereby promoting efficient SAPK pathway activation. The data in Figs. 7-14 demonstrate that by binding both MEKK1 and TRAF2, GCK may act to recruit MEKK1 to the TNFR signaling complex.

We next sought to determine the domains of GCK responsible for binding TRAF2. Cells were transfected with untagged TRAF2 and the indicated FLAG-tagged GCK constructs. FLAG isolates were probed with anti TRAF2 to detect the presence of associated TRAF2 (Fig. 15). From Fig. 15, it is clear that the CT and PEST1 domains of the GCK-CTD are required for binding TRAF1. Interestingly, CT is also required for binding MEKK1. We are investigating the possibility that TRAF2 may act to disinhibit GCK and foster MEKK1 binding.

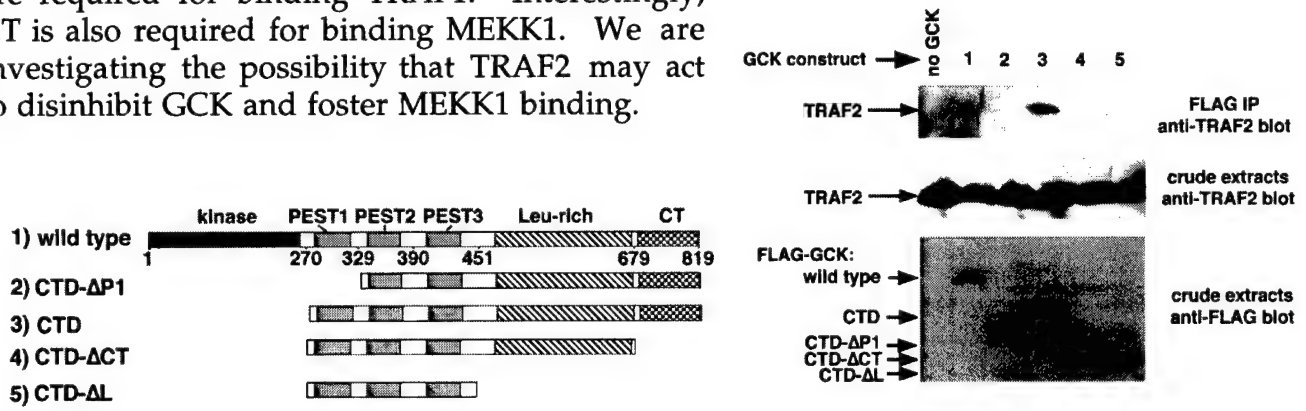

Fig. 15. GCK binding to TRAF2 requires the PEST1 and CT regions of the GCK-CTD. 293 cells were transfected with TRAF2 and the FLAG-tagged GCK constructs indicated in the left panel. Anti FLAG immunoprecipitates were probed with anti TRAF2 to detect TRAF2 associated with GCK. Numbers in the right panel indicate the GCK constructs used (shown in the left panel).

We wished next to determine the domains of TRAF2 that bind the GCK-CTD. TRAF2 consists of an amino terminal RING finger domain, five consecutive zinc finger domains and a carboxyterminal TRAF domain comprised of two tandem TRAF repeats (Fig. 16). 293 cells were transfected with FLAG-GCK-CTD and GST-tagged forms of the TRAF2 constructs indicated in the top panel of Fig. 16. It is clear that deletion of the TRAF domains abrogates completely GCK binding while deletion of the RING and/or Zn finger domains is without effect. Thus GCK binds TRAF2 through the Zn finger domain. By contrast, deletion of the RING finger domain results in a TRAF2 construct that cannot activate coexpressed SAPK. Thus, whereas TRAF domains may bind TRAF2 effectors, the RING domain mediates regulation of these effectors.

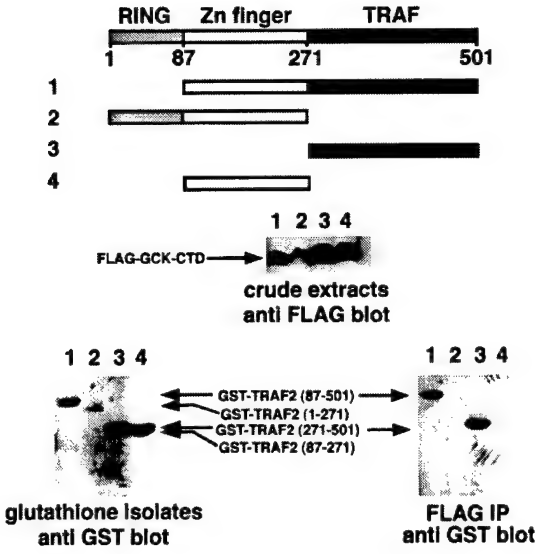

Fig. 16. The TRAF domains of TRAF2 mediate GCK binding. 293 cells were transfected with FLAG-GCK-CTD and GST-tagged forms of the TRAF2 constructs indicated in the top panel. Anti FLAG immunoprecipitates were probed with anti GST to detect bound TRAF2.

2.7 RIP Couples Both SAPK and p38 to the TNFR Signaling Complex: p38 Activation by RIP May Involve a MAP3K Associated with the RIP Intermediate Domain.

Our studies of RIP indicate that it can activate p38 and represents a redundant mechanism for SAPK activation that complements the TRAF2-GCK mechanism described above. Fig. 17 illustrates that the RIP-ID is necessary for activation of both SAPK and p38 by coexpressed RIP; and its deletion abrogates completely the ability of RIP to activate SAPK or p38. The subdomain of the RIP ID that mediates SAPK and p38 activation does not correspond to that (AAs 391-422 [60]) involved in NF-κB activation. The catalytic domain of RIP appears dispensable for SAPK and p38 activation by RIP inasmuch as the catalytic domain itself fails to activate SAPK or p38, and D138N-RIP, which is devoid of catalytic activity [60], can engender robust SAPK and p38 activation.

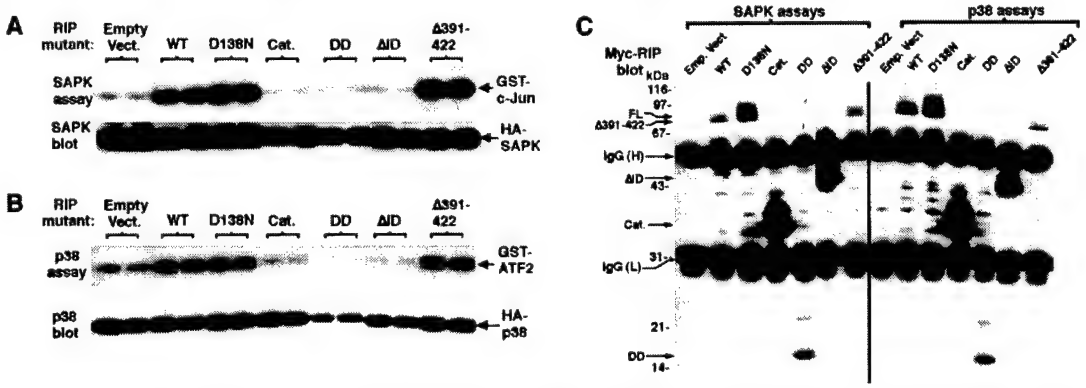

Fig. 17. The RIP ID is necessary for SAPK and p38 activation. The catalytic domain is dispensable. 293 cells were transfected with the indicated RIP constructs. SAPK (A), and p38 (B) were assayed using anti HA immune complexes. Myc-RIP constructs were immunoprecipitated and immunoblotted (C) to assess RIP expression. D138N is a kinase-inactive mutant, Cat, catalytic domain; DD, the RIP death domain; ΔID, deletion of the RIP intermediate domain; Δ391-422, deletion of the ID subdomain implicated [60] in NF-κB activation.

More detailed studies of the isolated RIP-ID indicate that it is sufficient for both SAPK and p38 activation. In addition, our findings indicate that deletion of the RIP-ID (RIP-ΔID) creates a dominant inhibitory molecule that can block SAPK and p38 activation

by TNF, likely by sequestering endogenous TRADD, or a related species that couples directly to TNFR1 [52,58-60] (Fig. 18)

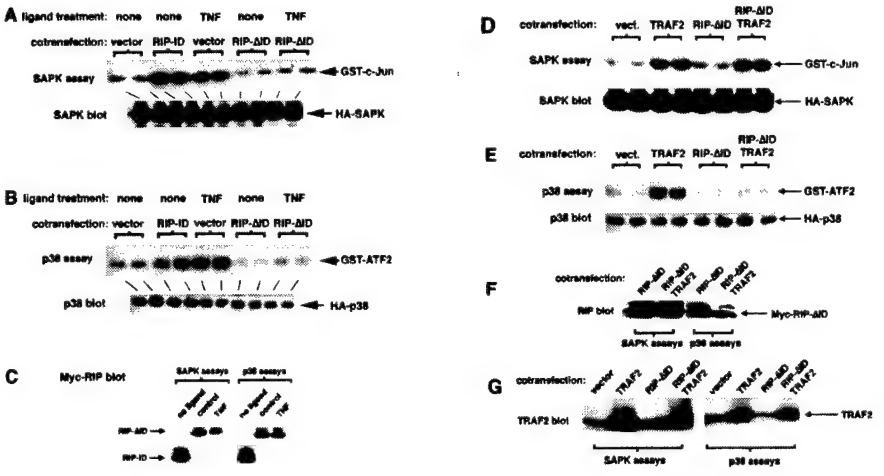

Fig. 18. The RIP ID is necessary and sufficient for RIP activation of the SAPKs. Requirement for the ID for TNF activation of the SAPKs (A-C). D-G show that TRAF2 signals through RIP to activate p38, but not to activate the SAPKs; thus the dominant inhibitory RIP-ΔID mutant blocks TRAF2 activation of p38 but not SAPK.

Fig 18 also shows that RIP is an effector for TRAF2 activation of p38, but not for TRAF2 activation of SAPK. Thus, the dominant inhibitory RIP-ΔID blocks TRAF2 activation of p38 but not of SAPK. The fact that SAPK activation by TRAF2 is not inhibited by RIP-ΔID might be explained by the observation that TRAF2 associates with other species upstream of the SAPKs, such as GCK (Fig. 14-16).

Figs. 8 and 9 indicate that RIP itself is not a MAP3K; and instead suggested to us that RIP might function in a manner analogous to that of GCK--as a binding protein for a MAP3K upstream of the SAPKs, p38s or both. To determine if RIP could associate with a MAP3K, we immunoprecipitated a spectrum of RIP mutants from transfected 293 cells and assayed for activation of the p38-specific MEK, MKK6 *in vitro*. The assay employed the telegraphic format similar to that first designed and used by the PI to assay the mitogenic MAP3K, Raf-1 [61]. Thus, RIP beads were incubated with purified, inactive MKK6 and ATP. A portion of the MKK6 was then removed and used to activate purified, inactive p38. The p38, in turn, was assayed for phosphorylation of ATF2. From the results in Fig. 10, it is evident that the RIP-ID can associate with a MAP3K capable of reconstituting the p38 pathway *in vitro*. The identity of this MAP3K is, at present, unknown. The MAP3K does not correspond to any of the known MAP3Ks upstream of the SAPKs or p38s. We have been unable to detect *in vitro* activation of SEK1 using this system. Thus RIP may not associate stably with a SAPK-specific MAP3K, or the MAP3K associated with RIP cannot activate SEK1 and, instead targets other SAPK-specific MEKs such as MKK7.

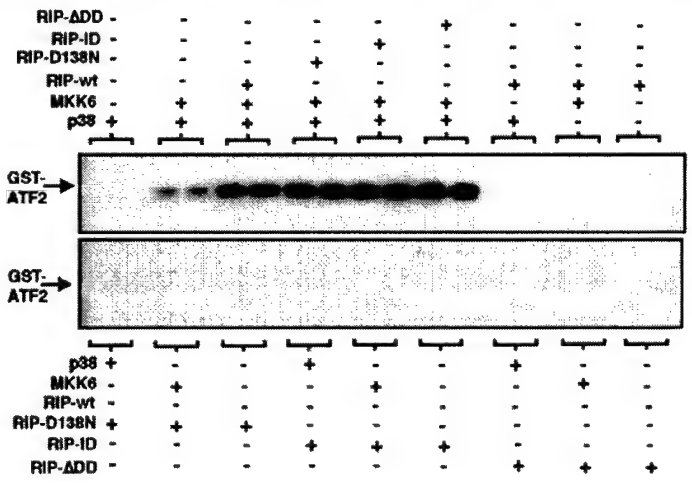

Fig. 19. *In vitro* activation of MKK6 by a MAP3K associated with the RIP-ID. RIP immunoprecipitates or nonimmune IPs were incubated with vehicle or MKK6 plus ATP as indicated. A portion of the MKK6/vehicle was removed and incubated with vehicle or p38 and ATP as indicated. The p38 was then assayed for ATF2 kinase activity. MKK6 and p38 were purified as GST-tagged proteins by GSH-agarose chromatography from extracts of transfected 293 cells, followed by elution with free GSH.

2.8 Regulation of Cell Cycle Progression by the Cdc42Hs → p38 pathway

In addition to characterizing the biochemistry of stress signaling, we sought to understand the biological functions of the various signaling components which we had identified. To this end we wished to develop a quantifiable bioassay system which could accurately reflect the biology of SAPK and p38 signaling elements. Microinjection proved the most ideal for this sort of study. High levels and efficiencies of expression were possible and the expressing cells could easily be counted for particular biological functions. Our initial studies employed a simple system, the cell division cycle of the NIH3T3 cell, which can easily be arrested in G0 by serum withdrawal. Cell cycle progression in these cells is also easily assayed using a variety of techniques. Our results, however, were quite unexpected, and point to the complexity of function of the SAPK and p38 pathways.

2.8.1 *Microinjection into growing cells of p38, but Not SAPK inhibits NIH3T3 cell cycle progression at G1/S*

Previous studies have indicated that the Ras-regulated MAPK cascade is required for cell growth and for entry from G0 into the cell cycle [36]. By contrast, many of the stimuli that activate the SAPK and p38 pathways (e.g., UV and γ radiation, chemical DNA damage) arrest cells already committed to the cell cycle at G1/S in order to allow for DNA and cellular repair [1,4,5,9]. Such cell cycle checkpoints are essential to cellular survival and to the prevention of cellular transformation in response to genotoxic agents. Accordingly, we wished to test directly if either the SAPK or p38 pathways could affect the cell cycle progression of cells already in G1. NIH3T3 cells were serum starved in order to arrest and synchronize the cell cycle at G0. Cells were then plated onto coverslips in 10% serum in order to re-initiate synchronously the cell cycle and were then microinjected, in early to mid G1, with expression plasmids encoding various signaling proteins. Cells expressing recombinant proteins (stained for FLAG or HA tags) were scored for G1/S transition by staining for bromodeoxyuridine (BrdU) incorporation.

We microinjected plasmids encoding p38, SAPK (p46-β1), p44 MAPK and p70 S6 kinase and observed that p38 expressed from microinjected plasmid markedly arrested cell cycle progression at G1/S (Fig. 20).

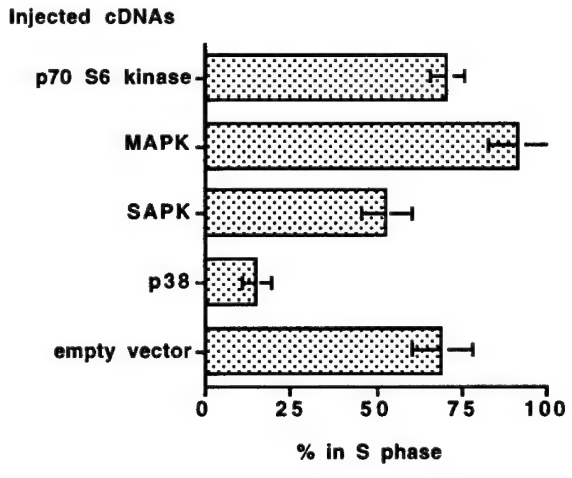

Fig. 20. Expression of microinjected p38 inhibits G1/S transition in NIH3T3 cells. Cells were synchronized and microinjected as described above. S phase entry was measured as described above.

Surprisingly, SAPK, which is activated by the same antimitogenic stimuli as p38 [1,4,5,9], did not significantly arrest cells in G1. Staining of microinjected cells for active p38 with an antibody against the phosphorylated, active form of p38 [1,6] revealed that microinjection increased the level of active p38 in the cells. Similarly, cells injected with SAPK, in spite of showing no growth inhibition, contained elevated levels of active SAPK as assessed by staining with an antibody specific for c-Jun phosphorylated by SAPK [1,4,5]. To confirm further that SAPK did not arrest cells in G1, we microinjected cells with GCK, a specific SAPK activator (Fig. 7). As can be seen in Fig.

21, GCK does arrest cells in G1. However, this arrest is likely due to the promiscuous activation of p38 which can occur upon massive GCK overexpression. In support of this, coinjection of GCK with a kinase-dead, dominant inhibitory MKK3 construct completely blocks GCK inhibition of G1/S transition, even if SAPK is coinjected.

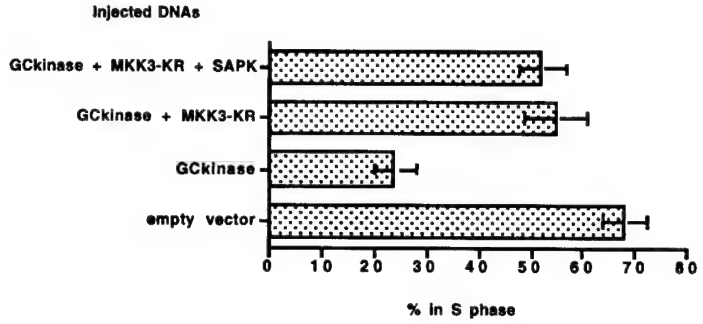

Fig. 21. SAPK is likely not able to arrest cells in G1. Inhibition of G1/S transition by GCK is due to nonspecific activation of p38. Microinjection experiments were performed as described. GCK was FLAG-tagged, SAPK and MKK3-KR were HA tagged.

2.8.2 *MEKs upstream of p38 inhibit G1/S progression*

To confirm that activation of p38 was a necessary step in G1/S arrest observed upon p38 microinjection, we tested three of the MEKs known to lie upstream of p38: SEK1, MKK3 and MKK6 [19,21,22]. All three of these MEKs were able to potently arrest cells at the G1 restriction point. Insofar as kinase-dead mutants of MKK3 or SEK were unable to arrest the cell cycle, it is clear that active MEKs and, therefore active p38 are necessary for G1 arrest (Fig. 22).

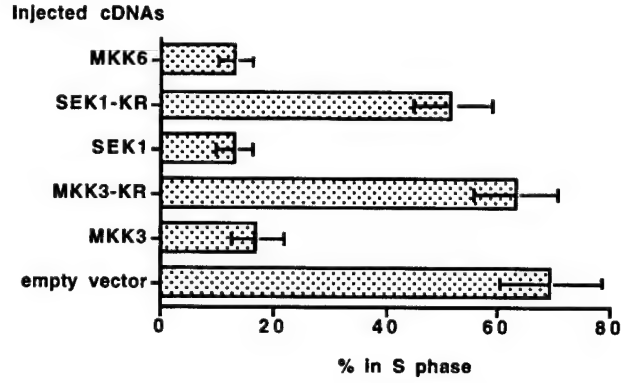

Fig. 22. MEKs upstream of p38 arrest cells in G1. inactive MEKs do not arrest the cell cycle. Cells were injected with the constructs indicated (all HA-tagged, except MKK6, which was FLAG tagged). G1 arrest was assessed as described above.

Further confirmation of the requirement for active p38 in cell cycle arrest came from the use of kinase-dead MEKs as inhibitors of activation of coinjected p38 [21,22]. In the experiment shown in Fig. 23, cells were microinjected with either p38 alone or p38 plus KR-MKK3 or SEK1. The kinase-dead MEKs were able to inhibit p38-mediated G1 arrest completely.

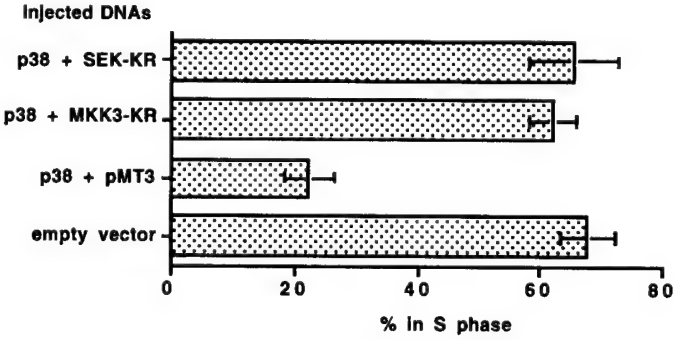

Fig. 23. Kinase-dead, dominant inhibitory mutants of MKK3 or SEK1 can inhibit p38-mediated G1 arrest. All injected constructs were HA-tagged. pMT3 is the empty parent vector used in this experiment. Staining with an antibody to p38 indicated that p38 was still overexpressed in the KR-MKK3 and KR-SEK-injected cells.

2.8.3 *The Rho family GTPase Cdc42Hs inhibits G1/S transition in part by activation of p38*

Both p38 and the SAPKs can be activated *in vivo* by Cdc42Hs and Rac1, two members of the Rho subgroup of the Ras superfamily [36-38]. To test if either of these

small GTPases could arrest the cell cycle, we microinjected expression plasmids encoding Cdc42Hs, Rac and, as a negative control, RhoA. As can be seen in Fig. 24, wild type Cdc42Hs, when expressed in cells in mid G1, strikingly inhibits cell cycle progression to S phase. By contrast, neither wild type nor a constitutively active allele of Rac1 (V12) markedly inhibits G1/S transition. Similarly, V12-RhoA, which does not activate SAPK or p38, does not block S phase entry. Moreover, the inhibition of cell cycle progression mediated by Cdc42Hs likely requires active p38 inasmuch as kinase-dead, dominant inhibitory MKK3 and SEK1 coinjected with Cdc42Hs block the ability of Cdc42Hs to arrest the cell cycle at G1.

These findings apparently contrast with earlier studies implicating Cdc42Hs in the program of Ras-dependent growth and transformation. In this regard, it should be noted that studies implicating Cdc42Hs in mitogenesis have employed *ras*-transformed cells, or have overexpressed Ras selectively in quiescent (G0) cells. By contrast, we have microinjected Cdc42Hs into cells in early- to mid-G1. Thus, our results indicate that the Cdc42Hs MKK3/6 p38 pathway may play a role in growth arrest of cells committed to the cell cycle, whereas Cdc42Hs be recruited as part of a mitogenic program in quiescent G0 cells.

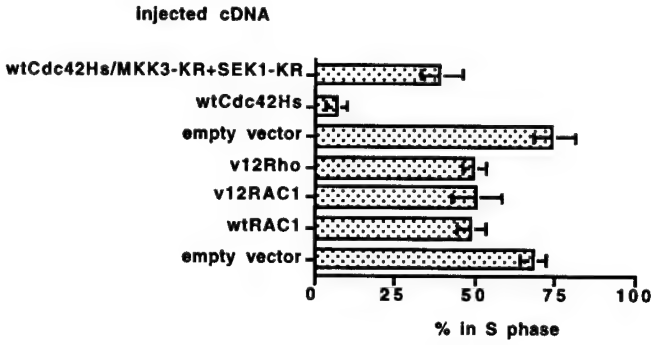

Fig. 24. Inhibition of G1 progression mediated by Cdc42Hs by a p38-dependent mechanism. All constructs injected were M2-FLAG-tagged except the KR-MEKs which were HA-tagged. Empty vector for the upper three bars was a combination of the FLAG and HA vectors (pCMV5 and pMT3). For the lower four bars, the empty plasmid injected was pCMV5. Activity of the Rac construct was confirmed by staining for membrane ruffling, a classical cellular response to Rac1 activation [36]. RhoA activity was monitored by staining for actin stress fibers in the injected cells [36]. NIH-3T3 cells were serum starved to synchronize in G0, replated on coverslips in the presence of serum and injected in early to mid G1. S phase entry was determined as above.

3. PUBLICATIONS

The following publications were supported by this grant

1. Kyriakis, J.M. and Avruch, J. (1995) S6 kinases and MAP kinases: Sequential intermediates in insulin/mitogen-activated protein kinase cascades. In: *Protein Kinases: Frontiers in Molecular Biology*, J.R. Woodgett, ed., Oxford University Press: Oxford.

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4. PERSONNEL

The following personnel were paid from this grant during the period 8/19/94-8/28/98:

John M. Kyriakis, Ph.D. Principal Investigator (8/94-8/98)
Anna Maria Forte, M.S. Research Technician (8/94-4/95)
Irma Sánchez, Ph.D. Post Doctoral Research Fellow (from 7/95-9/95)
Árpád Molnár, M.D. Post Doctoral Research Fellow (4/95-8/98)
Anthony Makkinje, Ph.D. Post Doctoral Research Fellow (3/96-7/96)
Takashi Yuasa, Ph.D. Post Doctoral Research Fellow (4/96-8/98)

5. CONCLUSIONS: 8/94-8/95

5.1 Studies of the Biochemistry of the TNF → SAPK Pathway

From the results presented above, we propose a model for TNF signaling which is shown below (Fig. 25). In this model, TRAF2 recruits both GCK and RIP. GCK, in turn, binds and participates in the regulation of MEKK1. MEKK1 activates selectively the SAPKs. RIP retains the potential to activate both the SAPKs and p38s, possibly by associating with a MAP3K upstream of MKK6 and p38, as well as, possibly, MKK7 or other SAPK-specific MEKs.

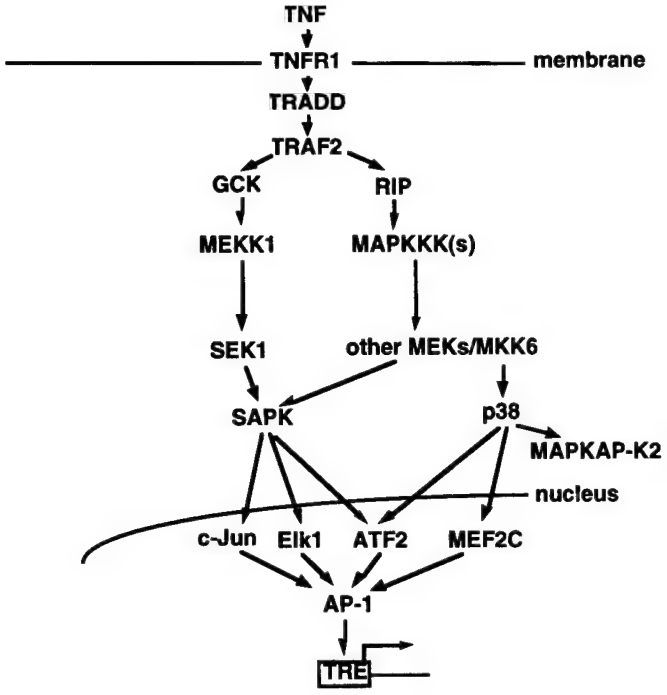

Fig. 24. Model for TNF signaling based on Preliminary Results. TRE is the TPA-response element which binds AP-1, triggering transcription.

5.2 Cell Biological Studies

Fig. 26 is a model that summarizes our results from the microinjection experiments.

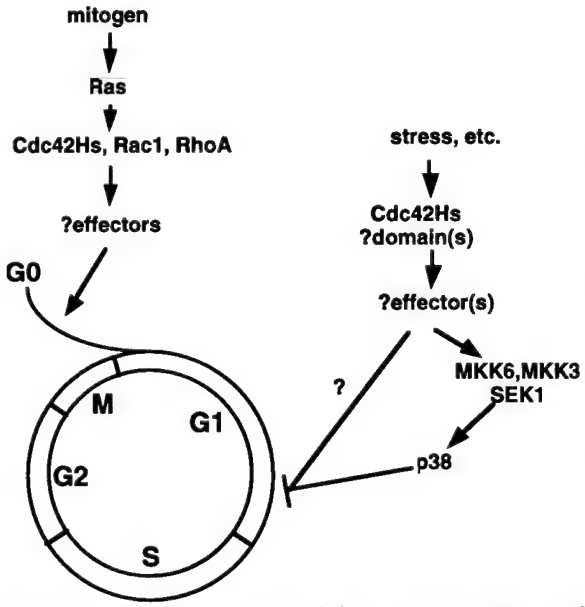

Fig. 26. Model of the effects of stress signaling on cell cycle.

We propose that activation of Cdc42Hs in actively growing cells (e.g., cells committed to the cell cycle) is antimitogenic and results in growth arrest G1/S. By contrast, activation of Cdc42Hs in quiescent cells, perhaps in concert with Rac1, as part of a Ras-dependent mechanism, results in cell cycle entry. Although Rac1 is clearly a component of Ras mitogenic signaling, both Rac1 and Cdc42Hs can activate p38; and activation of p38 appears to be obligatory for cell cycle arrest in response to activation of Cdc42Hs. Thus, it is likely that Cdc42Hs and Rac exert their effects on multiple pathways and the opposing effects of Cdc42 and Rac1 activation may involve these other pathways in addition to p38. What this means is that therapies which address p38 activation, including drugs developed from the novel pyridinyl imidazole p38 inhibitors (41), may not necessarily prevent and may actually promote breast cancer cell growth; and care must be taken to assess carefully the roles of these stress pathways in cell growth. Most importantly, the techniques and results summarized in Figs. 25 and 26 make possible the study of the role of these pathways in the growth of transformed breast cancer cells.

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